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Phosphorylated Claspin Interacts with a Phosphate-binding Site in the Kinase Domain of Chk1 during ATR-mediated Activation*

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Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated or UV-damaged DNA. The activated form of Claspin contains two repeated phosphopeptide motifs that mediate its binding to Chk1. We show that these phosphopeptide motifs bind to Chk1 by means of its N-terminal kinase domain. The binding site on Chk1 involves a positively charged cluster of amino acids that contains lysine 54, arginine 129, threonine 153, and arginine 162. Mutagenesis of these residues strongly compromises the ability of Chk1 to interact with Claspin. These amino acids lie within regions of Chk1 that are involved in various aspects of its catalytic function. The predicted position on Chk1 of the phosphate group from Claspin corresponds to the location of activation-loop phosphorylation in various kinases. In addition, we have obtained evidence that the C-terminal regulatory domain of Chk1, which does not form a stable complex with Claspin under our assay conditions, nonetheless has some role in Claspin-dependent activation. Overall, these results indicate that Claspin docks with a phosphate-binding site in the catalytic domain of Chk1 during activation by ATR. Phosphorylated Claspin may mimic an activating phosphorylation of Chk1 during this process.

Eukaryotic cells utilize checkpoint control mechanisms to preserve the integrity of their genomes (1, 2). For example, cells prevent the initiation of mitosis if DNA replication has not occurred normally. In vertebrates, this control system, which is known as the DNA replication checkpoint, employs the effector kinase Chk1 to suppress activation of the Cdc2-cyclin B complex, a critical regulator of the G_2/M transition (3). In particular, Chk1 down-regulates the phosphatase Cdc25 and up-regulates the kinase Wee1, which together control inhibitory phosphorylation of Cdc2 on tyrosine residue 15 (1). The activation of Chk1 in response to checkpoint-inducing signals in the genome is dependent on ATR, a protein kinase in the family that also includes ATM and DNA-PK (4–7).

We have been using *Xenopus* egg extracts to study how vertebrate cells monitor the presence of incompletely replicated DNA during the cell cycle. In this system, *Xenopus* Chk1 (Xchk1)[1] undergoes phosphorylation and activation in response to DNA replication inhibitors such as aphidicolin (8). Certain synthetic oligonucleotides also elicit the efficient activation of Xchk1 (9). The activation of Xchk1 requires phosphorylation on conserved Ser-Gln/Thr-Gln (SQ/TQ) motifs in its C-terminal domain by the *Xenopus* homologue of ATR (Xatr) (4, 5).

The Xatr-dependent phosphorylation and activation of Xchk1 depends upon Claspin, a large acidic protein that binds to Xchk1 during a checkpoint response (9). Homologues of Claspin called Mrc1 were identified subsequently in budding and fission yeast as regulators of the checkpoint effector kinases Rad53 and Cds1, respectively (10, 11). Likewise, Claspin shares functional properties with budding yeast Rad9 and fission yeast Crb2, which control the activation of Rad53 and Chk1, respectively (1). Claspin is also found in human cells, where it likewise functions as a critical component of the DNA replication checkpoint (9, 12). In recent studies, it was shown that Claspin interacts with chromatin in a highly regulated manner during S-phase (13). The chromatin binding properties of Claspin suggest that it acts as a checkpoint sensor protein that detects the presence of active DNA replication complexes in the nuclei of vertebrate cells (13). Mrc1 may fulfill a similar function in the yeast systems (14).

In previous structure-function studies, we identified properties of Claspin that allow it to interact with Xchk1 (15). Claspin contains a 57-amino acid segment, the Chk1-binding domain (CKBD), which is both necessary and sufficient for this interaction. The CKBD contains two repeated Chk1-binding (CKB) motifs that are approximately 10 amino acids in length. A serine residue in the center of each motif (Ser-864 and Ser-895) undergoes phosphorylation during a DNA replication checkpoint response. Phosphorylation of both Ser-864 and Ser-895 is necessary for efficient binding of Claspin to Xchk1, and this phosphorylation-dependent binding is essential for the activation of Xchk1 by Xatr. On the basis of these observations, we surmised that Xchk1 might contain a phosphate-binding site that would allow it to dock with Claspin. In this report, we have investigated the molecular features of Xchk1 that mediate its interaction with Claspin.

EXPERIMENTAL PROCEDURES

Production of Recombinant Xchk1 Proteins—A version of pFastBac encoding Xchk1 with GST and His_6 tags at the C-terminal end (Xchk1-GH) was described previously (9). To prepare the various truncation mutants of Xchk1-GH that are described in this study, the RsrII-SpeI fragment of pFastBac-Xchk1-GH was removed by digestion with RsrII and SpeI. DNA fragments encoding the desired sections of Xchk1 were amplified by PCR with the appropriate primers containing RsrII or SpeI sites. These fragments were ligated back into the digested pFastBac-Xchk1-GH vector. The N135A and 4AQ point mutants of Xchk1-GH

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[1] The abbreviations used are: Xchk1, *Xenopus* Chk1; Xatr, *Xenopus* homologue of ATR; CKBD, Chk1-binding domain; GST, glutathione S-transferase; MBP, maltose-binding protein; cAPK, cyclic AMP-dependent protein kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

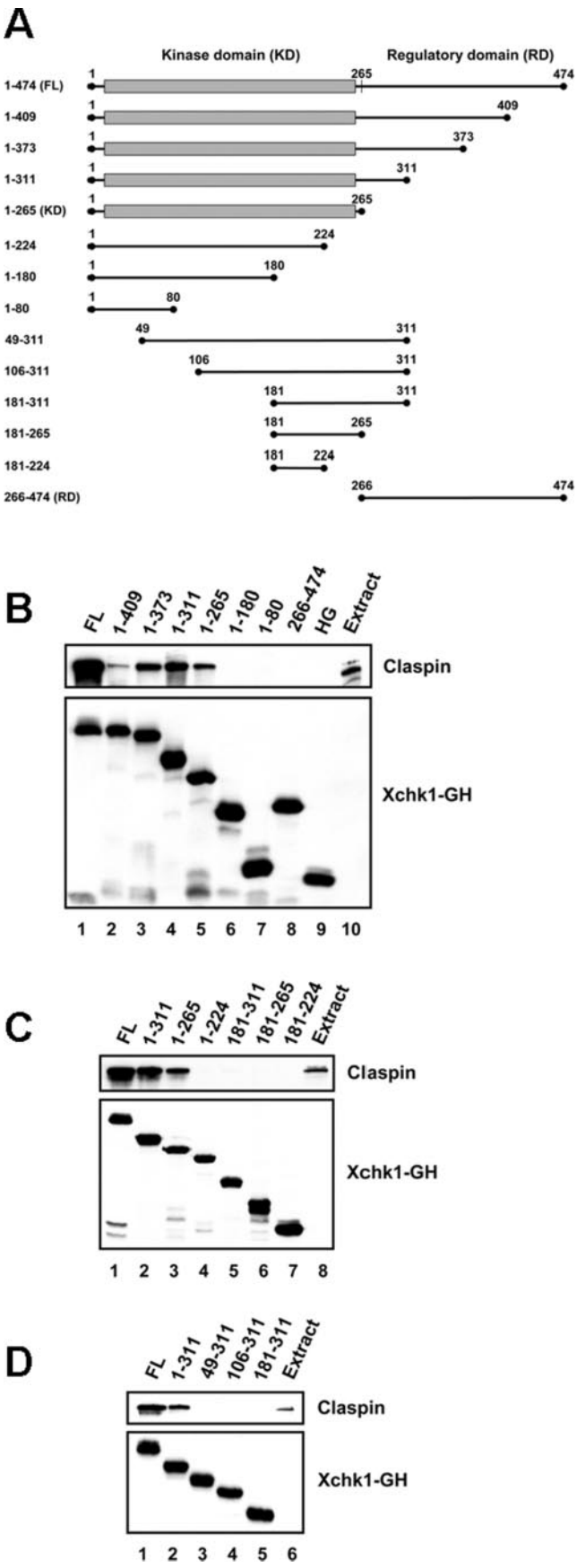

FIG. 1. **Claspin binds to the N-terminal kinase domain of Xchk1.** *A*, diagram of the various truncation mutants of Xchk1 used in this study. All polypeptides also contain GST and His_6 tags at the C-terminal end. *B*, full-length (*FL*) Xchk1-GH and the indicated fragments of Xchk1 (*lanes 1–8*) were incubated in egg extracts containing pA-pT and okadaic acid. His_6-GST (*HG*) was used as a control (*lane 9*). The tagged proteins were recovered, and binding of endogenous Claspin was determined by immunoblotting with anti-Claspin antibodies (*top*). Recovery of the tagged Xchk1 proteins was monitored by immunoblotting with anti-GST antibodies (*bottom*). *Lane 10* depicts the initial egg extract (1 μl). *C* and *D*, binding of Claspin to the indicated fragments from the kinase domain of Xchk1 was assessed as described in *B*.

were described before (9). The T377A and 4AQ/T377A mutants of Xchk1-GH were produced by swapping the appropriate DNA fragments from pFastBacHTa-Xchk1-T377A and pFastBacHTa-Xchk1–4AQ/T377A (16) into pFastBac-Xchk1-GH. The K53A, K54A, R129A, T153A, and R162A point mutants of Xchk1-GH were produced with the QuikChange kit (Stratagene). Recombinant baculoviruses were produced with the Bac-to-Bac system (Invitrogen). Recombinant proteins were expressed in Sf9 insect cells and purified as described previously (9). A ^{35}S-labeled fragment of Xchk1 containing amino acids 266–474 as well as Met and Ala at the N terminus was produced by using the TNT T7 Quick for PCR DNA kit (Promega) as described previously (15). For this procedure, the 5′ primer contained a T7 promoter sequence.

Preparation of Maltose-binding Protein (MBP)-CKBD—A DNA fragment encoding amino acids 838–920 of Claspin, which contains the CKBD, was amplified by PCR and cloned into pMAL (New England Biolabs) that had been digested with SalI and HindIII. MBP-CKBD was expressed in Codon-Plus (RIL) *Escherichia coli* cells, isolated with amylose resin (New England Biolabs), and eluted with HEPES-buffered saline containing 20 mM maltose.

Assay for Binding of Claspin to Xchk1 and Its Fragments—In one type of assay, recombinant Xchk1-GH and various mutants (1 μg) bound to nickel-agarose beads were added to 100 μl of *Xenopus* egg interphase extract containing 100 μg/ml cycloheximide, 3 μM okadaic acid, and 50 μg/ml of an annealed mixture consisting of poly(dA)$_{70}$ and poly(dT)$_{70}$, as indicated. The extracts were incubated for 100 min at room temperature under constant agitation. After the incubation, the beads were isolated by centrifugation and washed five times with buffer A (10 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 20 mM β-glycerol phosphate, and 0.5% Nonidet P-40). Alternatively, soluble Xchk1-GH and various mutants (1 μg) were incubated in egg extracts under the same conditions. Following the incubation, the extracts were diluted with 200 μl of buffer A and centrifuged through a 1-ml Sephadex G-25 column. The eluate was incubated with 10 μl of glutathione-agarose for 30 min at 4 °C. The beads were collected and washed three times with buffer A. Finally, all samples were subjected to SDS-PAGE.

Miscellaneous—^{35}S-Labeled wild-type Claspin and Claspin-2AG were produced with the TNT *in vitro* transcription/translation system (Promega) as described (15). Kinase assays for the activity of Xchk1 were performed with a GST-Cdc25C peptide as substrate as described previously (8). Immunoblotting with antibodies against Claspin, GST, and Ser(P)-344 of Xchk1 was described earlier (9, 13). Anti-MBP antibodies were purchased from New England Biolabs.

RESULTS

Claspin Interacts with the N-terminal Kinase Domain of Xchk1—In previous studies, we demonstrated that Claspin binds to a recombinant form of Xchk1 (Xchk1-GH) in *Xenopus* egg extracts containing an annealed mixture of poly(dA)$_{70}$ and poly(dT)$_{70}$ (hereafter called pA-pT) and a phosphatase inhibitor such as tautomycin (9). Subsequently, we have found that okadaic acid is comparably effective to tautomycin. We have used this assay to investigate what region of Xchk1 is necessary for binding to Claspin. Initially, we prepared various C-terminal truncations of Xchk1 (Fig. 1, *A* and *B*). We observed that truncations containing amino acids 1–409 and 1–373 of Xchk1 showed reduced binding to Claspin in comparison with full-length Xchk1 (residues 1–474). A more severe truncation containing amino acids 1–311 consistently bound to Claspin somewhat more efficiently than the 1–409 and 1–373 fragments. A further shortening of the 1–311 fragment to yield the minimal kinase domain (residues 1–265) resulted in diminished binding. Nonetheless, we found that the 1–265 fragment of Xchk1 bound well to Claspin under various assay conditions. As a control, we showed that a His_6-GST fusion protein alone could not bind to Claspin under these conditions. These exper-

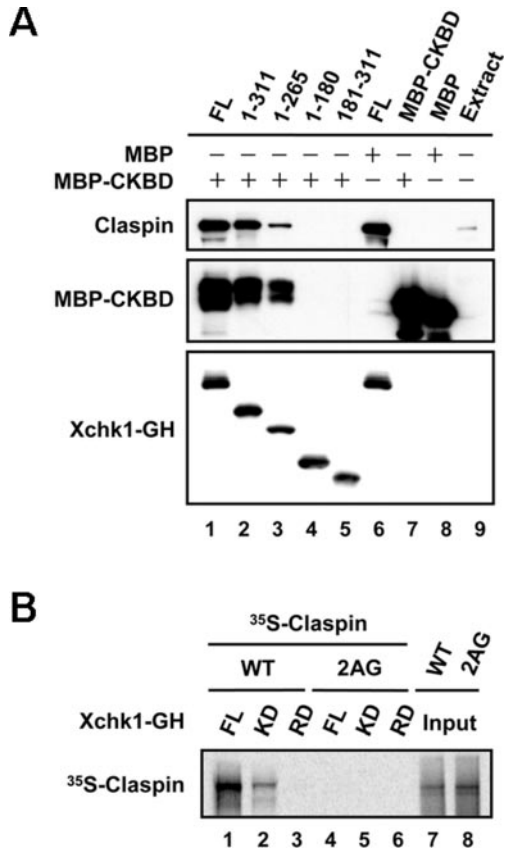

FIG. 2. **Binding of Claspin to the kinase domain of Xchk1 involves the CKBD and requires phosphorylation of Claspin on Ser-864 and Ser-895.** *A*, MBP-CKBD (*lanes 1–5*) and MBP alone (*lane 6*) were incubated in egg extracts containing full-length (*FL*) Xchk1-GH and the indicated fragments of Xchk1 in the presence of pA-pT and okadaic acid. The tagged Xchk1 proteins were reisolated, and the samples were processed for immunoblotting with antibodies against Claspin (*top*), MBP (*middle*), and GST (*bottom*). *Lanes 7* and *8* depict the initial preparations of MBP-CKBD and MBP, respectively. *Lane 9* depicts untreated egg extract (1 μl). *B*, ^{35}S-labeled versions of wild-type Claspin (*lanes 1–3*) and Claspin-2AG (*lanes 4–6*) were incubated in egg extracts containing full-length Xchk1-GH (*lanes 1* and 4), Xchk1-(1–265)-GH (*lanes 2* and *5*), and Xchk1-(266–474)-GH (*lanes 3* and *6*) in the presence of pA-pT and okadaic acid. The tagged Xchk1 proteins were reisolated, and binding of the labeled Claspin proteins was assessed after gel electrophoresis and detection with a PhosphorImager. *Lanes 7* and *8* depict the input ^{35}S-labeled proteins. *KD*, kinase domain; *RD*, regulatory domain.

iments indicate that Claspin clearly interacts with the kinase domain of Xchk1 (residues 1–265). Nonetheless, removal of all or part of the C-terminal regulatory domain of Xchk1 (residues 266–474) reduces the binding of Claspin.

Next, we sought to identify the location in the kinase domain that interacts with Claspin (Fig. 1, *C* and *D*). As one approach, we prepared a variety of C- and N-terminal truncations of the kinase domain (residues 1–265). In these experiments, we were unable to identify any fragment of Xchk1 smaller than the minimal kinase domain that could interact detectably with Claspin (Fig. 1, *C* and *D*). These results indicate that Xchk1 most probably must possess an intact and properly folded kinase domain to associate with Claspin.

Interaction of Claspin with the Kinase Domain of Xchk1 Involves the CKBD—In parallel, we asked whether the binding of Claspin to the kinase domain of Xchk1 involves the mechanism that we previously identified for interaction with full-length Xchk1. We first examined whether a polypeptide containing the CKBD from Claspin could bind to the kinase domain of Xchk1 (Fig. 2*A*). For this experiment, we prepared a fusion protein that contains the MBP and residues 838–920 of Claspin. We incubated the MBP-CKBD in egg extracts containing full-length Xchk1-GH, Xchk1-(1–311)-GH, or Xchk1-(1–265)-GH. We observed that all three Xchk1 polypeptides bound to the MBP-CKBD with relative efficiencies that were comparable with what we had observed for binding to full-length Claspin. In a control incubation, MBP alone did not interact with Xchk1 in this assay. We also asked whether the binding of Claspin to the kinase domain of Xchk1 requires phosphorylation on Ser-864 and Ser-895 of Claspin. For this purpose, we incubated ^{35}S-labeled versions of full-length, wild-type Claspin or the Claspin-2AG mutant (which contains alanine at positions 864 and 895) in egg extracts with full-length Xchk1-GH, Xchk1-(1–265)-GH, or Xchk1-(266–474)-GH in the presence of pA-pT. As shown in Fig. 2*B*, full-length Xchk1 and the 1–265 fragment bound to wild-type Claspin but not to the Claspin-2AG mutant. There was no detectable binding of the 266–474 fragment of Xchk1 to either form of Claspin. Taken together, these results indicate that the kinase domain of Xchk1 binds to the CKBD of Claspin in a manner that requires phosphorylation on Ser-864 and Ser-895.

The Kinase Domain of Xchk1 Contains a Phosphate-binding Site That Interacts with Phosphorylated Claspin—To define more precisely how Claspin interacts with the kinase domain, we attempted to identify positively charged residues (*e.g.* lysine and arginine) on the surface of Xchk1 that could potentially form a phosphate-binding site for the CKB motifs (Figs. 3 and 4). The crystal structure of residues 1–289 from human Chk1 has been solved to atomic resolution (17). A notable feature of this structure is that it contains a sulfate ion near the catalytic center of Chk1. There are numerous examples in which sulfate ions mimic phosphate groups and associate with phosphate-binding sites in proteins during crystallization procedures (17). In the case of human Chk1, the sulfate ion resides in a positively charged cluster of amino acids that contains Lys-54, Arg-129, Thr-153, and Arg-162 (17).

For illustration, we have diagramed how these residues could interact with a phosphate group (see Fig. 4). Interestingly, these amino acids all sit in regions of Chk1 that are generally important for various aspects of catalytic function in a wide variety of kinases (18, 19). For example, Arg-129 precedes Asp-130, which is a highly invariant residue in the catalytic loop of protein kinases (Fig. 3*A*). The corresponding amino acid in the cyclic AMP-dependent protein kinase (cAPK) is Asp-166. This residue acts as the base that removes a proton from the hydroxyl group of the protein kinase substrate. Thr-153 and Arg-162 lie in a section of Chk1 that corresponds to the activation loop in various kinases. In the cases of cAPK and Cdk2, for example, this loop undergoes a phosphorylation that helps to position the critical aspartate residue in the catalytic loop. Finally, Lys-54 precedes Glu-55 in the αC helix of Chk1. The equivalent glutamic acid in the αC helix of cAPK is Glu-91, which forms a ion pair with Lys-72 and thereby helps to stabilize interactions between Lys-72 and the α and β phosphates of ATP. Significantly, the position of the sulfate ion in the crystal structure of human Chk1 corresponds to the location of the activating phosphorylation in cAPK and Cdk2 (18, 20). Paradoxically, however, Chk1 appears not to undergo this type of activating phosphorylation on its polypeptide chain (17).

We mutated Lys-54, Arg-129, Thr-153, and Arg-162 individually to alanine in the full-length Xchk1-GH protein. We observed that none of the K54A, R129A, T153A, or R162A mutants could associate stably with Claspin under the conditions of our binding assay (Fig. 3*B*). For comparison, we mutated Lys-53, which is a positively charged residue that lies immediately adjacent to Lys-54, but does not face the putative phos-

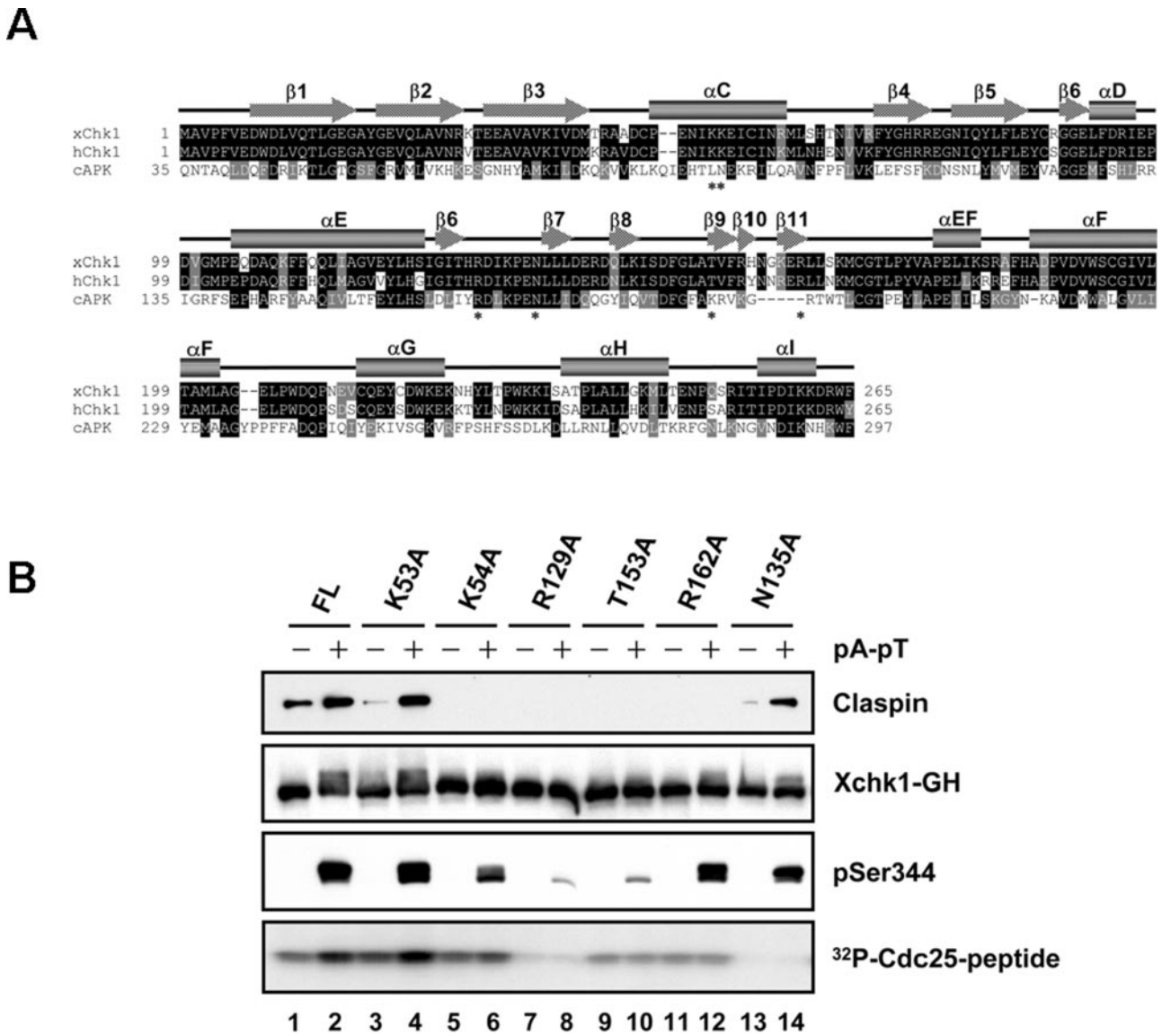

FIG. 3. **Effects of point mutations in the kinase domain of Xchk1 on binding to Claspin.** *A*, sequence alignment of the kinase domains from Xchk1, human Chk1, and mouse cAPK. Lys-53, Lys-54, Arg-129, Asn-135, Thr-153, and Arg-162 of Xchk1 are denoted with *asterisks*. *B*, wild-type Xchk1-GH and various mutant forms of Xchk1 with the indicated alterations in its kinase domain were incubated in egg extracts in the absence (*lanes 1*, *3*, *5*, *7*, *9*, *11*, and *13*) or presence (*lanes 2*, *4*, *6*, *8*, *10*, *12*, and *14*) of pA-pT. All samples contained okadaic acid. The tagged Xchk1 proteins were reisolated. In some cases, the samples were processed for immunoblotting with antibodies against Claspin (*top panel*), GST (*second panel from top*), and Ser(P)-344 of Xchk1 (*third panel from top*). For the *bottom panel*, the various reisolated Xchk1 proteins were assayed for kinase activity toward a GST-Cdc25C peptide. The samples were subjected to gel electrophoresis, and ^{32}P incorporation was detected with a PhosphorImager.

phate-binding site. We found that the K53A mutant of Xchk1 binds very well to Claspin. We also investigated the Claspin binding properties of a previously characterized kinase-inactive mutant of Xchk1 (N135A) in which Asn-135 has been changed to alanine (9). Although Asn-135 also resides in the catalytic center of Chk1, we observed that the N135A mutant interacts efficiently with Claspin.

To pursue these observations further, we asked whether mutants of Xchk1 with alterations in the putative phosphate-binding site could undergo Xatr-mediated phosphorylation and activation (Fig. 3*B*). For this analysis, we examined the phosphorylation of Ser-344 on Xchk1 in the absence and presence of pA-pT. Ser-344 is a major site on Xchk1 for phosphorylation by Xatr (4). We also assayed the kinase activity of Xchk1 toward a GST-Cdc25C peptide (8). In the case of the R129A and T153A mutants, we observed that the checkpoint-dependent phosphorylation of Xchk1 on Ser-344 was almost completely abolished. The R129A mutant displays negligible kinase activity in both the absence and presence of pA-pT. The T153A mutant possesses a basal kinase activity similar to that of wild-type Xchk1. However, the T153A mutant, in contrast to wild-type Xchk1, does not undergo an increase in activity in response to pA-pT.

In the case of the K54A mutant, there was reduced but detectable phosphorylation on Ser-344. Likewise, activation of the K54A mutant in the presence of pA-pT was diminished substantially but not abolished. Finally, the R162A mutant underwent significant phosphorylation on Ser-344, but this protein did not undergo any increase in activity in the presence of pA-pT. Thus, for the R162A mutant, there appears to be an uncoupling between phosphorylation on Ser-344 and elevated kinase activity. Although we could not detect binding of the K54A and R162A mutants to Claspin, these mutants may still be capable of interacting transiently with Claspin. Perhaps this transient binding would be sufficient to enable some phosphorylation of Xchk1 by Xatr. Overall, these experiments indicate

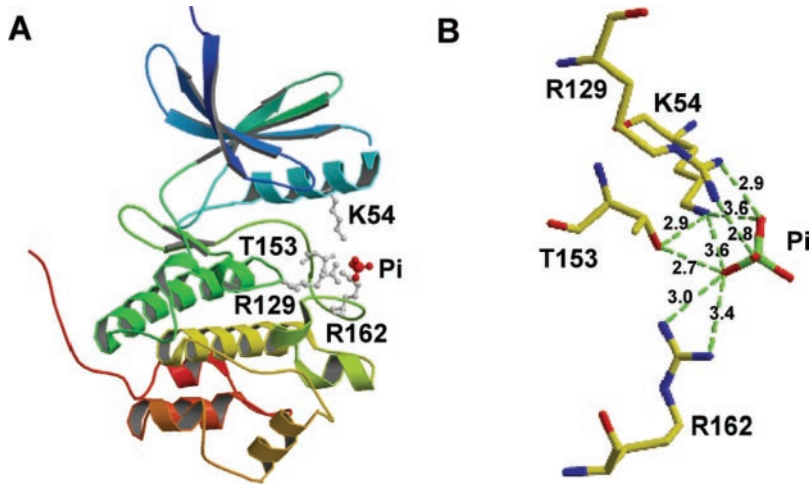

FIG. 4. **Illustration of the putative phosphate-binding site in Chk1.** *A*, ribbon diagram of residues 1–289 from human Chk1. The side chains for Lys-54, Arg-129, Thr-153, and Arg-162, which are conserved in Xchk1, are indicated. The putative position of the phosphate group from a CKB motif of Claspin is illustrated. This position corresponds to that of the sulfate group in the crystal structure of human Chk1-(1–289) (17). Atomic coordinates were obtained from the Protein Data Bank (accession code 1IA8). *B*, stick diagram of Lys-54, Arg-129, Thr-153, and Arg-162 and potential interactions with a phosphate group. Distances are indicated in angstroms.

that residues in the phosphate-binding site of Xchk1, especially Arg-129 and Thr-153, are important for the Claspin-dependent activation of Xchk1.

The C-terminal Regulatory Domain of Xchk1 Requires Claspin for Its Phosphorylation—Although Claspin binds to the N-terminal kinase domain of Xchk1, the C-terminal domain of Xchk1 also appears to have some role in regulating the interaction with Claspin. For example, removal of all or part of the C-terminal regulatory domain from Xchk1 significantly diminishes binding to Claspin (Fig. 1*B*). This observation raises the possibility that the C-terminal domain of Xchk1 might contain a second binding site for Claspin or might contribute to the Claspin-binding site in the N-terminal kinase domain. However, we have not been able to observe stable binding of Claspin to the isolated C-terminal domain of Xchk1 (see Fig. 1*B*). To investigate this issue further, we examined the checkpoint-dependent phosphorylation of the C-terminal regulatory domain of Xchk1 in the presence and absence of Claspin (Fig. 5). Consistent with previous results (21), we observed that a ^{35}S-labeled fragment of Xchk1 containing its C-terminal regulatory domain (amino acids 266–474) underwent phosphorylation in the presence of pA-pT and okadaic acid. Significantly, this phosphorylation was abolished in extracts from which Claspin had been removed by immunodepletion. Furthermore, the checkpoint-dependent phosphorylation of the Xchk1-(266–474) fragment could be restored by the addition of recombinant His$_6$-Claspin to the Claspin-depleted extracts. The phosphorylation of the Xchk1-(266–474) fragment still occurred in extracts from which the endogenous full-length Xchk1 protein had been immunodepleted (not shown). Therefore, the phosphorylation of the Xchk1-(266–474) fragment cannot be facilitated by association with endogenous full-length Xchk1. Overall, these observations suggest that Claspin also may associate transiently with the C-terminal regulatory domain of Xchk1.

Claspin Dissociates from the Fully Activated Form of Xchk1—To characterize the interaction between Claspin and Xchk1 in further detail, we examined the binding of Claspin to the activated, highly phosphorylated form of Xchk1. In one set of experiments, we examined the time course of binding between Claspin and Xchk1 in egg extracts containing pA-pT and okadaic acid. As shown in Fig. 6*A*, the binding of Claspin to Xchk1 was maximal at around 70 min. In these extracts, the most highly phosphorylated form of Xchk1 appeared at 100–130 min. This observation suggests that the fully phosphorylated form of Xchk1 may dissociate from Claspin.

As another means to examine this issue, we treated egg

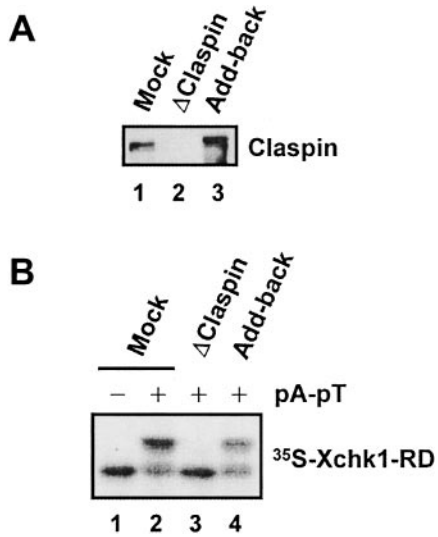

FIG. 5. **The C-terminal regulatory domain of Xchk1 depends upon Claspin for its phosphorylation.** *A*, immunodepletion of Claspin. Egg extracts were treated with control (*lane 1*) or anti-Claspin (*lanes 2* and *3*) antibodies (9). For *lane 3*, recombinant His$_6$-Claspin was added back to the extract at a final concentration of 40 μg/ml. Extracts were immunoblotted with anti-Claspin antibodies. *B*, the extracts shown in *A* were incubated in the absence (*lane 1*) or presence (*lanes 2–4*) of pA-pT, as indicated. The extracts also contained an exogenously added ^{35}S-labeled version of the regulatory domain of Xchk1 (residues 266–474). After a 100-min incubation, the mobility of Xchk1 was monitored by SDS-PAGE and phosphorimaging.

extracts with pA-pT and okadaic acid to trigger the phosphorylation of Claspin and then added recombinant Xchk1-GH and EDTA to these extracts (Fig. 6*B*). As expected, Xchk1 does not undergo phosphorylation in the presence of EDTA. Nonetheless, the hypophosphorylated form of Xchk1 in EDTA-containing extracts binds very well to the Claspin that had undergone phosphorylation prior to the addition of EDTA. By comparison, in the absence of EDTA, we observed considerably less binding of fully phosphorylated Xchk1 to Claspin. These findings are also consistent with the concept that hypophosphorylated Xchk1 binds preferentially to Claspin and then dissociates as a consequence of phosphorylation and activation.

Previously, we observed that point mutations in the TRF motif in the C-terminal half of Xchk1 (*e.g.* T377A) result in a constitutive 15-fold elevation in kinase activity relative to wild-type Xchk1 in egg extracts that lack a checkpoint-inducing DNA signal (16). Therefore, we considered the possibility that forms of Xchk1 with a mutated TRF motif might display al-

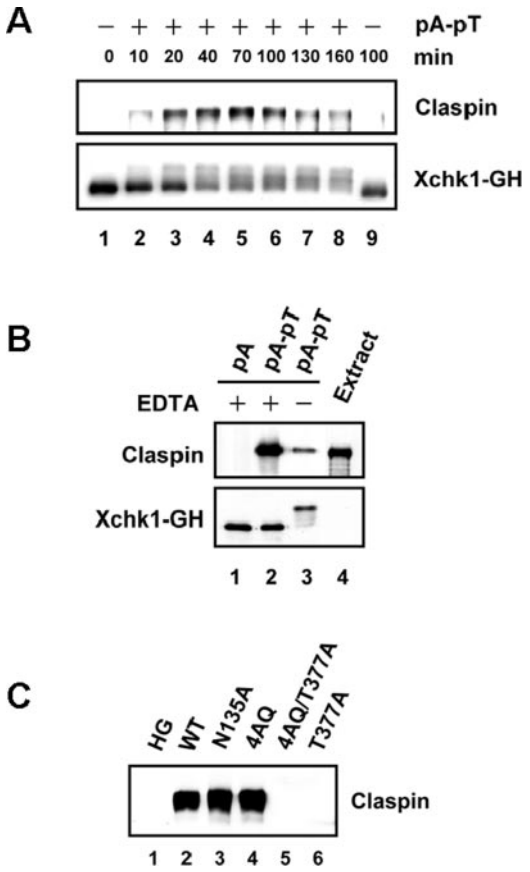

FIG. 6. **Claspin dissociates from the fully activated form of Xchk1.** *A*, time course of interaction between Claspin and Xchk1 in checkpoint-activated extracts. Recombinant Xchk1-GH was incubated in egg extracts in the absence or presence of pA-pT. At the indicated times, the tagged Xchk1 was reisolated, and binding of Claspin was determined by immunoblotting with anti-Claspin antibodies (*top*). Xchk1-GH was detected with anti-GST antibodies (*bottom*). *B*, Claspin interacts strongly with the hypophosphorylated form of Xchk1. At time 0, egg extracts (100 μl) were incubated with either pA (*lane 1*) or pA-pT plus okadaic acid (*lanes 2* and *3*) for 90 min at room temperature. At this time, 200 μl of a solution containing 20 mM EDTA, 0.1% CHAPS, 2.5 mM EGTA, and 20 mM β-glycerol phosphate in HEPES-buffered saline solution was added to the samples (*lanes 1–3*). Xchk1-GH was added either at time 0 (*lane 3*) to allow its modification in the extract or after addition of the EDTA-containing buffer (*lanes 1* and *2*) to prevent its phosphorylation. Thereafter, Xchk1-GH was re-isolated through a 60-min incubation at 4 °C with glutathione-agarose beads. The bead preparations were subjected to SDS-PAGE and probed by immunoblotting with anti-Claspin (*top*) and anti-GST antibodies (*bottom*). *Lane 4* depicts untreated egg extract. *C*, the constitutively activated, hyperphosphorylated T377A mutant of Xchk1 does not bind well to Claspin. His_6-GST (*lane 1*), wild-type (*WT*), full-length Xchk1-GH (*lane 2*), and various point mutants of Xchk1-GH (*N135A*, *4AQ*, *4AQ/T377A*, and *T377A*) (*lanes 3–6*) were incubated in egg extracts containing pA-pT and okadaic acid. The tagged proteins were reisolated, and binding of Claspin was determined by immunoblotting with anti-Claspin antibodies. Immunoblotting with anti-GST antibodies indicated that recovery of His_6-GST and the tagged Xchk1 proteins was similar for all samples (not shown).

tered Claspin-binding properties. To investigate this matter, we assayed the Claspin-binding ability of the T377A mutant of Xchk1 (Fig. 6*C*). In addition, we examined both the 4AQ mutant of Xchk1, which cannot serve as a substrate for Xatr, and the combined 4AQ/T377A mutant (4, 16). The 4AQ/T377A mutant displays similarly elevated activity as the T377A single mutant. We observed that there was negligible binding of the Xchk1-T377A mutant to Claspin in comparison with wild-type Xchk1. Moreover, the 4AQ mutant of Xchk1 bound well to Claspin, whereas the combined 4AQ/T377A mutant displayed no association with Claspin. Thus, a point mutation in the C-terminal regulatory domain of Xchk1 that increases its kinase activity in the absence of a checkpoint signal also abolishes binding to Claspin. This abolition of binding occurs even if Xchk1 cannot undergo phosphorylation of its SQ/TQ motifs by Xatr. Taken together, all of the above results suggest that Claspin has a lower affinity for activated Xchk1 than for inactive Xchk1.

DISCUSSION

In this report, we have investigated the features of Xchk1 that allow it to interact with Claspin during a checkpoint response. Through deletion analysis, we found that the N-terminal kinase domain of Xchk1 (residues 1–265) can interact specifically with Claspin in checkpoint-activated extracts. By constructing various point mutants of Xchk1, we identified four residues in the kinase domain (*e.g.* Lys-54, Arg-129, Thr-153, and Arg-162) that are involved in the interaction with Claspin. Interestingly, these particular residues comprise a sulfate-binding site in the crystal structure of human Chk1 (17). This observation raised the possibility that these amino acids might normally be involved in coordinating a phosphate group in the cell. In previous studies, we established that, during a checkpoint response, Claspin becomes phosphorylated on two sites (Ser-864 and Ser-895) that reside in the center of two repeated peptide sequences in the CKBD of Claspin. As shown here, phosphorylation of Ser-864 and Ser-895 of the CKBD is necessary for stable binding of Claspin to the isolated kinase domain of Xchk1. These results suggest that the phosphopeptide motifs in the CKBD of Claspin interact with the phosphate-binding pocket in Xchk1. In addition, we have obtained evidence that the C-terminal domain of Xchk1 (residues 266–474) has a role in regulation by Claspin.

As an overall model, the activated form of Claspin that has undergone phosphorylation on Ser-864 and Ser-895 docks with Xchk1 by means of the phosphate-binding site in its kinase domain of Xchk1 (Fig. 7). The presence of two CKB motifs in Claspin might allow as many as two molecules of Xchk1 to dock with each molecule of Claspin. By examining the structure of the kinase domain from human Chk1 (17), we inferred that the spacing between the CKB motifs of Claspin most likely would enable the binding of two Xchk1 molecules to a Claspin monomer. However, we do not have direct evidence that two molecules of Xchk1 can bind to one Claspin protein. Other possibilities are that Xchk1 may contain a second binding site for the CKB motifs or that two CKB motifs may allow Claspin to interact more avidly with a single binding site in the Xchk1 monomer.

Because Claspin binds to Xchk1 and is necessary for the Xatr-mediated activation of Xchk1, a simple model is that Claspin somehow recruits Xatr to Xchk1. The docking of the CKB motifs of Claspin onto the kinase domain of Xchk1 may facilitate this recruitment and thereby promote the phosphorylation of Xchk1 by Xatr. If two Xchk1 molecules can indeed bind to one molecule of Claspin, these interactions might also facilitate intermolecular autophosphorylation of Xchk1. Once Xchk1 becomes activated, our results suggest that Xchk1 would dissociate from Claspin. As described in this paper, the hypophosphorylated, low-activity form of Xchk1 binds better to Claspin than the hyperphosphorylated, activated version of Xchk1. This dissociation of activated Xchk1 would allow Claspin to recruit more Xchk1 for additional rounds of activation. By analogy, it has been shown in budding yeast that the hypophosphorylated, inactive form Rad53 interacts best with Rad9, whereas the activated, hyperphosphorylated version of Rad53 dissociates from Rad9 (22).

The interaction between Claspin and Xchk1 bears some similarities with the glycogen synthase kinase-3β system (23).

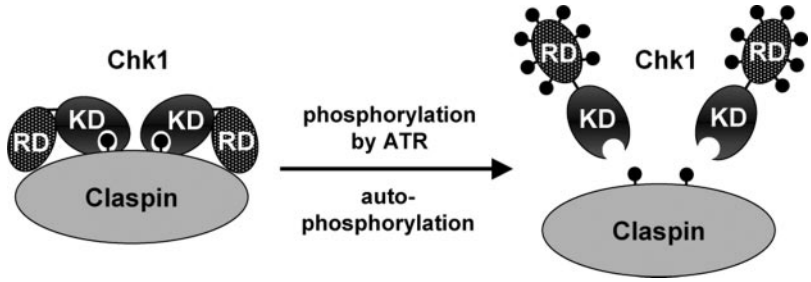

FIG. 7. **Model for the interaction of Xchk1 with Claspin.** Claspin, which has been phosphorylated on its two CKB motifs, recruits one or more molecules of Xchk1. Phosphorylation of Xchk1 on its SQ/TQ motifs by Xatr and autophosphorylation lead to the activation of Xchk1. The fully phosphorylated form of Xchk1 dissociates from Claspin.

Glycogen synthase kinase-3β utilizes a phosphate-binding site in its kinase domain to dock with prospective substrates that have already received a priming phosphorylation. In humans, for example, the priming phosphate group on a substrate interacts with glycogen synthase kinase-3β by means of Arg-96, Arg-180, and Lys-205 in the kinase domain. The positions of these residues correspond to those of Lys-54, Arg-129, and Thr-153 in Xchk1, respectively. Thus, both Xchk1 and glycogen synthase kinase-3β employ a phosphate-interacting pocket in their kinase domains to recognize other molecules. However, a significant difference between the two systems is that Claspin is an upstream regulator and not a substrate of Xchk1. In this regard, we have not been able to observe *in vitro* phosphorylation of Claspin by Xchk1.[2]

An interesting question is why Claspin would dock with Xchk1 near its catalytic center. A significant observation from structural studies is that the kinase domain of human Chk1 (residues 1–289) adopts an open lobe conformation with a slight misalignment of residues that are critical for catalysis (17). The structure of the 1–289 fragment from human Chk1 does not vary with and without a bound sulfate ion (17), suggesting that a phosphate group alone at this location would not significantly affect the conformation of the kinase domain. The consensus sequence for the CKB motif (E*XXX*LCSG*X*F) is highly conserved in metazoan Claspin molecules. It is possible that residues in addition to the phosphoserine in this motif might interact with side chains of Xchk1 in the vicinity of the phosphate-binding site. It is conceivable that such interactions might alter the conformation of Xchk1 so that it could achieve its most active state. Alternatively, this interaction may simply allow docking between Claspin and Xchk1.

[2] S.-Y. Jeong, A. Kumagai, J. Lee, and W. G. Dunphy, unpublished data.

In conclusion, we have found that Claspin interacts with Xchk1 by means of a phosphate-binding site in its kinase domain. This step is important for the proper activation of Xchk1 during a checkpoint response.

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